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(54) Title: TRANSFORMATION OF PLANTS BY DIRECT INJECTION OF DNA

(57) Abstract

A microinjection technique for direct transformation of plants. The method involves injection of exogenous DNA into a fertilized plant ovule at the onset of embryogenesis.

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TRANSFORMATION OF PLANTS BY DIRECT INJECTION OF DNA

Government Support

The present invention was made with the assistance of Grant No. 90-34133-5476 from the United States Agriculture Department. The government has certain rights in the invention.

Field of the Invention

This invention relates to transformation of plants, including sunflower, Helianthus annuus L. by direct injection of DNA into a fertilized ovule at the onset of embryogenesis.

Background of the Invention

Plant transformation has been achieved through various means. The first to be developed and most frequently used employs Agrobacterium tumefaciens as a vector to introduce alien DNA into the genome of the targeted plant. This technique was initially limited to the host plants of A. tumefaciens (i.e. dicotyledons), but was recently successfully applied to a monocot, corn, Zea mays L., [(Gould et al., Plant Physiol., 95, 426-434 (1991)]. However, success of the technique remains dependent upon the ability to regenerate plants from infected explants. For most important crops, this ability has not been developed or is not compatible with the needs of the transformation technique through A. tumefaciens.

Other currently known techniques for transformation, include electroporation and bombardment with DNA-coated microprojectiles. In the case of electroporation, the technique has only been successful for species that can be regenerated from protoplasts.

Microprojectile bombardment to introduce alien DNA into the genome of a host plant is exemplified by Dupont's Biolostic Particle Delivery System. [See also e.g. Sanford et al., Part. Sci. and Techn., 5, 27

(1987); European Patent Application No. 331,855 Sanford et al.] In soybean, Glycine max L., bombardment of over 20,000 shoot-apices only resulted in transient expression of the transforming gene whereas bombardment of embryogenic suspensions with the same gene produced several transformed plants (Buising et al., Proc. 3rd Biennal Conf. on Molecular and Cellular Bio. of the Soybean, July 1990; Finer and McMullen, Proc. 3rd Biennal Conf. on Molecular and Cellular Bio. of the Soybean, July 1990).

Current techniques of plant transformation, i.e. electroporation and microprojectile bombardment require the ability to regenerate plants from single cells. For most crops, this ability has yet to be developed despite repeated efforts. These techniques also require sophisticated technical skills and facilities.

Accordingly, there is a need for a technique of plant transformation with fewer requirements that will be applicable to most crops, easy to implement and cost effective. There is a need for a technique of direct transformation that eliminates the need for a vector, such as A. tumefaciens to introgress foreign DNA into the genome of a host plant and that also eliminates the need to regenerate plants from cultured explants.

Summary of the Invention

The present invention relates to a method for transformation of plants by direct injection of exogenous DNA into the endosperm of a fertilized plant ovule at the onset of embryogenesis. Preferably, the exogenous DNA sequence will correspond to a desired physical or functional characteristic to be imparted to the host plant. The DNA sequence is introgressed into the host plant genome, thus producing a transformed plant embryo.

According to the present invention, DNA can be injected into a fertilized plant ovule at a preglobular stage, globular stage, or heart-shaped stage of plant embryos. Example times for injection of exogenous DNA into a fertilized plant ovule is at about 12-96 hours after host plant fertilization.

Subsequent to the injection of the exogenous DNA into their endosperm, the fertilized plant ovules are cultured on a growth medium to develop embryos capable of growing into transformed plants on the same media. Preferably, these ovules are positioned horizontally on the growth medium employed for culturing.

According to the present invention, injection of exogenous DNA is accomplished using a tapered needle that is inserted into the endosperm through the butt end of the ovule, about two-thirds of the way through the ovule towards the embryo. In a preferred embodiment, an approximately 1 μl plasmid solution having about 1 μg/μl to 1 ng/μl of plasmid DNA is injected into the endosperm of the host plant ovule.

The method of the present invention is particularly well suited for transformation of sunflower, Helianthus annuus L. However, the present invention is believed to be applicable to dicotyledonous and monocotyledonous plants in general and could be employed for direct transformation of wheat, soybean and corn ovules, for example. The method of the present invention provides for direct transfer of exogenous DNA to a host plant ovule at the onset of embryogenesis and in vitro culturing of ovules that produces transformed plants without the need for a vector or the regeneration of plants from cultured explants.

Brief Description of the Drawings

Fig. 1 shows identification of β-glucuronidase in leaf extracts of putative transgenic plants (purple coloration) [c1: 150 units of β-glucuronidase; c2: 15 units of β-glucuronidase; and c3: 1.5 units of β-glucuronidase].

Fig. 2 shows autoradiograms showing presence of plasmid pBI221 in leaf DNA of putative transgenic plants (T) [P: control plasmid].

Fig. 3 shows histochemical identification of the GUS-gene in putative transgenic plants (blue spots).

Fig. 4 shows electrophoresis and autoradiograms of PCR products from DNA amplification of transgenic plant progenies [lane 1: 1 kb DNA ladder].

Detailed Description of the Invention

The present invention is directed to a method for producing transformed plants, including dicotyledonous and monocotyledonous plants including all species of sunflowers, e.g. Helianthus annuus L., soybeans, e.g. Glycine max L., corn, e.g. Zea mays, wheat, e.g. Triticum aestivum or the like. As described herein the present invention provides for the direct transfer of exogenous DNA to host plants and integration of the exogenous DNA into the host plant genome. Progenies of the transgenic plants inherit the extrachromosomal DNA.

As used herein the term "heterologous, exogenous, or foreign DNA" refers to DNA originating from a source outside the host or recipient plant. The term "DNA segment" refers to a DNA sequence having a nucleotide base composition and length capable of being introgressed into the genome or a gene complex of a host plant. The term "host plant" refers to a plant chosen to receive an exogenous DNA segment. The term "gene" refers to a segment of DNA composed of a transcribed region and a regulatory sequence that makes

transcription possible and can, either alone or in combination with other genes, provide the organism with an identifiable trait.

The term "trait" refers to detectable physical or functional characteristic of an organism. The term "phenotype" is a particular manifestation of a trait which corresponds to the presence of a particular gene. The term "homology" refers to two regions of DNA which contain regions of nearly identical DNA sequences. "Transformant" refers to a host plant which has been transformed with genetic material according to the methodology of the invention. Similarly, "transgenic" is used herein to include any plant, plantlet, or fertilized ovule the genotype of which has been altered by the presence of "heterologous, exogenous, or foreign DNA," wherein the DNA was introduced into the genome by the described genetic engineering process according to the invention, or which was initially introduced into the genome of a parent plant by the process of the present invention and is subsequently transferred to later generations by sexual crosses or asexual propagation.

As used herein "genome" refers to the sum total of hereditary genetic material within a cell's chromosomes. The term "heritable" means that the DNA is capable of transmission through at least one complete sexual cycle of a plant, i.e., it is passed from one plant through its gametes to its progeny plants. As used herein, the term "plasmid" refers to autonomously replicating extrachromosomal DNA which is not integrated into a microorganism's genome and is usually circular in nature.

The method of this invention produces transformed plants by (i) introducing exogenous genetic material into a fertilized plant ovule of a host plant at the onset of embryogenesis in a manner providing for introgression of the genetic material into the genome of

the fertilized ovule of the host plant, and (ii) culturing the fertilized ovule on growth medium to maximize generation of transformed plantlets capable of developing into transformed plants which themselves can transmit to their progeny a trait resulting from the presence of the exogenous DNA in the host plant genome.

Plant Species and Development Stage for Transformation Process and Embryogenesis

The method of the invention is believed to be applicable to a wide range of plants including species of wheat, soybean, corn, or the like. The method is particularly useful to produce transformed sunflower plants. In the case of plants to be transformed according to the invention, as evidenced by sunflower, introduction of an exogenous DNA segment is made at the onset of embryogenesis inside a fertilized plant ovule.

It is believed that one of skill in the art will recognize the onset of embryogenesis of a fertilized ovule in plants. For sunflower, this occurs from about 4 hours after fertilization and extends until about 24 hours when the fertilized ovule begins to swell and darken. The method of the invention can be employed when the fertilized ovule is in the preglobular, globular or heart-shaped stage of embryo development. For sunflower, exogenous DNA introduction is preferably made from about 12 hours to about 96 hours after ovule fertilization. It is to be understood that globular and heart-shaped stages of development may be reached faster or slower depending on the particular plant or crop, and growing conditions in the greenhouse or growth chamber.

In sunflower, the method can be used to introduce exogenous DNA into ovules having embryos not yet visible with the naked eye or dissecting scope. However, the method is more effective when DNA is introduced into ovules in which the embryo is somewhat visible with a dissecting scope; that is, globular stage

(less than about 0.1 mm) or when the DNA is introduced into ovules in which the embryos are visible with the dissecting scope; that is, heart-shaped stage (0.1-0.5 mm).

Exogenous DNA For Transformation

The exogenous DNA that is introgressed into the host plant genome can be derived or isolated from any source, and may be chemically altered prior to introduction into a fertilized plant ovule. By way of example, the exogenous DNA can be an identified useful fragment from a plant other than the host plant, and which is chemically synthesized in pure form. The DNA of interest can be removed from the source of interest by chemical means such as using restriction endonuclease, so that it can be further modified if desired by amplification and methodology of genetic engineering.

The exogenous DNA can be completely synthetic DNA, semi-synthetic DNA, DNA isolated from non-host plant biological sources, or the like. For example, the exogenous DNA includes but is not limited to DNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses, modified genes, portions of genes, and chimeric genes of diverse origin including plants other than the host plant.

The exogenous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by regulatory sequences which promote the expression of the exogenous DNA present in the transformed plant. For example, the exogenous DNA may itself comprise or consist of a promoter that is active in the host plant or may utilize a promoter already present in the host plant genome.

The compositions of, and methods for, constructing DNA segments which can transform certain plants are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful in the invention. The present invention is not directed to the specific exogenous DNA and is not dependent upon the exogenous DNA composition used.

It is to be understood that the exogenous DNA will in many cases be a plasmid-borne gene for a recognizable phenotypic trait. However, other suitable DNA segments for use in the present invention include selectable marker genes, reporter genes, enhancers, introns, and the like. It is also envisioned that the exogenous DNA can be a sequence that expresses, inhibits, enhances, or modifies biological activity when introgressed into the host plant genome and when coupled to an appropriate targeting sequence such as a homologous insertion sequence. Suitable methods of construction for exogenous DNA segments are provided in the literature [See J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2d ed., 1989)]. Generally, the exogenous DNA will be relatively small such as less than about 30 kb. Relatively short sequences are preferred because they minimize susceptibility to physical, chemical, or enzymatic degradation which increases as the size of the DNA increases.

Exogenous DNA useful in the invention includes DNA which provides for, or enhances, a beneficial feature of the resultant transformed plant. The DNA may encode proteins or antisense DNA transcripts in order to promote increased food values, (e.g. increased essential amino acid content) higher yields (increased lysine production), pest resistance (e.g. Bacillus thuringiensis, endotoxin), disease resistance, herbicide

resistance, and the like or to modify the host plant physiology (reduction of flower abortion in soybean).

Microinjection of DNA

An important aspect of the invention is the manner for delivery of exogenous DNA into the fertilized plant ovule. Unlike known techniques of plant transformation, the invention employs microinjection of exogenous DNA into the endosperm of a fertilized plant ovule. The DNA is delivered, preferably through a small volume (e.g., less than 5 μl) capillary tube that has a needle-like tapered end, preferably about 1 μl [about 0.04 mm]. The exogenous DNA is preferably in a biologically suitable solution, such as TE(10 mM Tris-HCl pH 8.0/1 mM EDTA pH 8.0). When the exogenic DNA is in the form of a plasmid, the amount of plasmid in solution can vary from about 2 μg/μl to 1 ng/μl. The pressure applied during DNA injection and the pressure within the ovule must be appropriate to accomplish exogenous DNA incorporation. In that regard, the amount of DNA solution injected into the ovule should be between about 15 percent to 30 percent of the volume of that ovule. Preferably, the volume of exogenous DNA-containing solution should be about 1 μl for sunflower ovules. While the amount of exogenous DNA can vary the amount of exogenous DNA solution should not increase the volume of the ovule excessively to avoid rupture.

The invention involves exogenous DNA injection into the fertilized ovule endosperm. Preferably, the injection is made from the butt end of the ovule opposite the micropyle end. It has also been found preferable to insert the needle through the butt end about two-thirds of the way into the ovule through the area of the endosperm membrane/megasporangium, and into the endosperm.

Embryo Culturing and Growth

Subsequent to the introduction of exogenous DNA into the fertilized plant ovule endosperm, the developing embryos are preferably cultured in vitro on a suitable growth medium. The growth medium will of course vary depending on the particular plant. It is envisioned that the medium composition will be standard growth medium as understood by those of skill in the art. For example, the following can be used: salt solutions of Murashige and Skoog [Physiol. Plant, 15, 473-497 (1962)], Gamborg et al. [Exp. Cell Res., 50, 151-158 (1968)], and Nitsch and Nitsch [Science, 163, 85-87 (1969)] supplemented with Gamborg's vitamins, 3% sucrose and 0.1 mg/L naphtalene acetic acid, discussed in 1991 Crop Science, 31(1), 102-108 (1991) are incorporated by reference herein.

It has been found preferred that fertilized ovules be horizontally positioned on the medium during in vitro culturing. The time and temperature for culturing the fertilized ovules will also vary depending on the plant. In the case of sunflower, fertilized ovules will be grown for about two weeks at about 25°C under about 16 hours of light per day.

After an appropriate in vitro culture period, plant embryos are extracted from the ovule and grown on fresh growth media of the same composition into plantlets. Plantlets are subsequently transferred to pots and grown to maturity. Some seeds from mature transformed plants possess the heritable genetic information corresponding to the exogenous DNA introduced to the fertilized host plant ovule.

Establishment of Transformation

Evidence of host plant transformation and introgression of exogenous DNA can be determined using known histological, molecular, and immunological techniques. Such techniques as understood by those

skilled in the art are represented by the techniques employed in the Example.

Uses of Transformed Plants

Transformed plants produced by the method of the invention are expected to be useful for a variety of commercial and research purposes. Transformed plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g., agronomic traits such as pest resistance, herbicide resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g., improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g., improved processing traits).

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissues from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules.

Two specific uses of the invention are to supplement the nutritional value of sunflower by introgressing genetic material for soybean seed storage proteins into sunflower and increase sunflower genetic resistance to insects by introgressing toxin genes into sunflower.

The following example illustrates the present invention. It is presented to better explain the general procedures which were used to prepare a transformed sunflower which can express and transmit introgressed exogenous DNA to progeny.

Example

Histochemical, immunologic and molecular evidence described herein shows that transgenic plants of sunflower were produced through microinjection of foreign DNA at the onset of embryogenesis into fertilized ovules of sunflower cultured in vitro.

Material

Three public cytoplasmic male sterile inbred lines of sunflower, cmsHA99, cmsHA290 and cmsHA300, and one South Dakota line, cmsSDB861206, were used as sources of fertilized ovules. Two public restorer lines, RHA297 and RHA299, were used as sources of pollen. Two plantings (January and April) of 7 to 10 plants from each genotype were greenhouse grown in 152-mm plastic pots containing 2:1:1 (v:v:v) soil/peat moss/perlite under 16-hour light photoperiod (provided by two sodium vapor and two mercury lamps) and 20°C minimum night temperature. Heads from the female lines were hand-pollinated [twice] with pollen from the two restorer lines when stigmata were receptive. Pollination times were recorded as indicated below.

Ovule and Embryo Culture

Ovaries were collected in March for the January planting (series I) and in June/July for the April planting (series II) for two days after hand-pollination, and surface-sterilized 10 minutes in a 40% commercial bleach solution with Tween 80 (12 drops for 200 ml).

Specifically, hand-pollination with fresh pollen of RHA 297 and RHA 299 was done in the afternoon twice when stigmata of female lines were receptive. Three collecting times were employed:

I: 16 to 20 hours after hand-pollination;
II: 40 to 44 hours after hand-pollination; and
III: 64-68 hours after hand-pollination.

The collecting requirements were as follows:

1. Stigmata were brown and shrivelled otherwise ovaries were not collected. These two traits indicated that fertilization more likely occurred.
2. Ovaries were darkening (grayish rather than whitish), swollen and with thickened walls. These characteristics indicate that ovaries have been fertilized and that their ovules carried one embryo.

Embryo development in collected ovaries was as follows:

Stage I ovaries:	Embryos not visible with naked eye or dissecting scope. They were at best globular.
Stage II ovaries:	Embryos somewhat visible with a dissecting scope. They were at the globular stage (about 0.1 mm).
Stage III ovaries:	Embryos visible with a dissecting scope. They were at the heart-shaped stage (0.1-0.5 mm).

Ovules (20-75) were excised aseptically and planted on three different media in 15 x 100 mm petri dishes sealed with parafilm. These media were MS (Murashige and Skoog, supra, 1962), B5 (Gamborg et al., supra, 1968), NN (Nitsch and Nitsch, supra, 1969) supplemented with 0.1 mg/l naphthalene acetic acid and 3% sucrose, and solidified with 0.8% agar. Both treated and control ovules were grown for 14 days on the three media under 16-hour cool white light and 25°C ± 2°C. Ovules were then opened, developing embryos were excised and grown on the same fresh medium for germination into small plantlets. Plantlets were transferred onto the same media in baby food jars and greenhouse planted in

pots in commercial Sunshine mix when both shoots and roots were sufficiently developed (3 to 5 cm long). Seeds were recovered from most plants upon selfing. The results are for the three media confounded.

Transformation Procedure

Plasmid pBI221 was purchased from CloneTech (Palo Alto, CA) (CloneTech 1992/1993 Catalog, p. 174, and GUS Gene Fusion System User's Manual, incorporated by reference herein). It contains a 3 kb insert composed of the CaMV 35S promotor, β-glucuronidase gene (GUS gene), and Nopaline Synthase polyadenylation region on a HindIII-EcoR1 fragment of pUC19. The plasmid was amplified in E. coli:DH10B™ [purchased from Bethesda Research Laboratories (BRL), Gaithersburg, MD] according to the manufacturer procedure. More specifically, this procedure involved the following materials and protocol:

MATERIAL:

E. coli: DH10B™ competent cells from BRL
This strain has a high efficiency of transformation and carries the mcrBC$^-$ and mrr^{--} deletions that minimize elimination of inserted methylated DNA (stored at -70°C).

Plasmid: pBI221 (2.5 μg/5 μl) from CloneTech carrying the GUS gene (3 kb) on a HindIII-EcoR1 fragment of pUC19

MEDIA/SOLUTIONS:

SOC

Bactotryptone	2 g
Yeast Extract	0.5 g
NaCl	1 ml from a 1 M stock solution (5.844 g/100 ml)
KCl	0.25 ml from a 1 M stock solution (7.455 g/100 ml)
Millipore water	99 ml

Stir, autoclave for 20 min. at 15 psi, cool to room temperature and add

1 ml from a 2 M Mg^{++} solution (20.333 g $MgCl_2$ + 12.04 g Mg SO_4/100 ml)
1 ml from a 2 M glucose solution (36.04 g/100 ml)
(both solutions are filter-sterilized through 0.2 µm millipore syringe filter unit; rinse the filter units first with sterile water)

Adjust pH to 7.0
Filter-sterilize with millipore filter (0.2 µm)

LB

Bactotryptone	10 g
Yeast Extract	5 g
NaCl	5 g
Millipore water	1000 ml
Agar	15 g

Autoclave for 20 min. at 15 psi, cool to 50°C, add 10 ml filter-sterilized ampicillin solution (100 mg) for a final concentration of 100 µg/ml (rinse 0.2 µm millipore syringe filter-unit with sterile water)

PROCEDURE

DAY ONE

Thaw competent cells and plasmid on wet ice

Dilute plasmid in TE so that 5 µl = 0.05 ng

Place 4 microcentrifuge tubes on ice

first tube: 20 µl of competent cells

second tube: 40 µl of competent cells + 5 µl pUC 19 (0.05 ng)

third tube: 40 µl of competent cells + 5 µl pBI 221 (0.05 ng)

fourth tube: 5 µl plasmid alone (0.05 ng) in 40 µl TE

Incubate 30 min. on ice

Heat shock in 42°C water bath for 45 seconds

Incubate 2 min. on ice

Add 0.960 ml SOC at room temperature

Incubate 1 hr. at 37°C in water bath

Spread 100 µl of each tube on LB + ampicillin agar plate

Incubate overnight at 37°C in dark

DAY TWO

Determine transformation efficiency:

$$\frac{\text{number of colonies}}{\mu\text{g of plasmid used}} \times \frac{\mu\text{l}}{\mu\text{g}} \times \text{dilution}^*$$

*v plated/v total

Isolate one colony from LB + ampicillin plate and streak on fresh plate for purity screening.

Incubate at 37°C overnight in dark

DAY THREE

Collect one colony from streak plate to inoculate 5 ml LB + ampicillin broth (optimum 1 colony for 10 ml of medium)

Grow suspension at 37°C from 6 hrs. to overnight under shaking conditions

Pipet 4 x 1 ml of that suspension to inoculate 4 x 250 ml of LB + ampicillin in 1000 ml flask (optimum culture volume to flask volume: 1:10 to 1:30)

Grow overnight at 37°C under vigorous shaking

After amplification, the pBI221 plasmid was extracted according to the Alkaline extraction procedure stated in Berger and Kimmel, Methods in Enzymology, 152, 166-167 (1987), incorporated by reference herein. This procedure employed:

SOLUTIONS

TRIS-GLUCOSE-EDTA

25 mM Tris-HCl pH 8

50 mM filter-sterilized glucose (to prevent spheroplasts from lysis)

10 mM EDTA

LYSOZYME STOCK SOLUTION (denatures outer cell wall, spheroplast formation)

400 mg/10 ml of sterile millipore water

NaOH-SDS

0.5 ml of 20 N NaOH (to raise pH to 12.0 to 12.5)

500 mg SDS (to complete cell lysis)

49.5 ml of sterile millipore water

PROCEDURES

Pellet transformed bacteria by centrifuging liquid culture 10 min. at 5000 rpm (use centrifuge bottles, do not exceed 5000 rpm)

Discard supernatant

Cell Lysis

Resuspend pellets in 20 ml of Tris-glucose-EDTA

Add lysozyme (2 mg/ml or 1 ml of 40 mg/ml solution)

Divide into four 50 ml tubes

Incubate on ice for 10 min.

Alkaline Extraction - prepurification - Procedure

Add 10 ml NaOH-SDS solution to each tube

Incubate on ice for 10 min.

Add 7.5 ml of 3 M sodium acetate solution (pH 4.8) to each tube

Chill in freezer for 15 min.

Centrifuge at 10,000 rpm for 15 min.

Phenol Extraction

Transfer supernatant to cloudy 250 ml centrifuge bottles

Add equal volume of phenol/chloroform (1:1)

Mix vigorously

Centrifuge at <5000 rpm for 5 to 10 min.

Pipet supernatant to clean cloudy 250 ml centrifuge bottles

Add equal volume of chloroform

Mix vigorously

Centrifuge at <5000 rpm for 5 to 10 min.

Pipet supernatant to 50 ml tubes

Ethanol Precipitation

Add 0.1 volume of 3 M sodium acetate (pH 4.8)

and 2 volumes of ice-cold ethanol

Split into several tubes

Place at -20°C

The pBI221 plasmid was then purified by EtBr/CsCl density gradient using the procedure of Berger and Kimmel, supra (1987), incorporated by reference herein. This procedure involved:

MATERIAL

DNA solution in ethanol at 20°C

Centrifuge at 10,000 rpm for 10 min.

Pour off supernatant

Drain pellet

PROCEDURE

Resuspend pellet in TE (recommended DNA concentration 50-100 µg/ml in 4 ml) so that 4 ml contain:

3.7 ml of TE

0.3 ml of Et-Br (10 mg/ml)

Add 1 g of Cesium Chloride per ml of DNA solution

Shake well

Transfer solution to Beckman Quickseal tubes (tubes must be almost full)

Ultracentrifuge at 50,000 rpm for >20 hrs. at 25°C

Observe bands in UV light. In case of plasmid purification, two bands should be present, the lower band being the plasmid. The upper band is the bacteria chromosomal DNA.

Make one hole at the top of the tube to eliminate pressure

Remove lower band with 18G needle attached to a 3 ml syringe (twist needle under band to penetrate the tube, suck as little as possible, put tape to avoid shearing)

Add equal volume of H_2O-saturated 1-butanol (1:1 in volume, two phases)

Remove upper phase with pasteur pipet and discard in special container

Repeat until all pink has been removed and then one more time

Add 3 volumes of water (to avoid CsCl to precipitate)

Add 2.5 volumes of ice-ethanol

Precipitate on ice or in freezer for 1 hr. or longer

Centrifuge 10 min. at 10,000 rpm

Pour off supernatant very carefully

Wash pellet with 70% ethanol

Drain and dry under vacuum

Resuspend in desired volume of TE

Store at 4°C

Several concentrations of pBI221 (from 1 ng/μl to 2 μg/μl) [1 ng/μl, 1 μg/μl and 2 μg/μl] were directly injected inside the ovule using a hand-made glass needle having a tapered end. Control ovules were injected with 1 μl TE buffer or left untouched.

The needle used to inject ovules was a 3 μl handmade needle made from a 5 μl capillary tube that was heated on an alcohol lamp, then drawn thinner under a dissecting microscope. The needle was inserted into the butt end of the ovule, two-thirds straight inside the ovule, to reach the endosperm-containing embryo sac of the ovule. Where the needle is located can be seen by transparency in the light.

The plasmid solution is delivered into the endosperm containing "embryo sac" of the ovule using a wire plunger that pushed the solution inside. Only 1 μl of plasmid solution was injected. Larger amounts (e.g.,

2 µl) caused the ovule to burst. Using this method a large number of ovules (400 or greater) can be plated and treated in a working day by an individual.

Plating of Injected Ovules

Ovules were laid down, that is horizontally positioned on top of the media. The culture media used were the salt solutions of Murashige and Skoog, supra (1962), of Gamborg et al., supra (1968), and of Nitsch and Nitsch, supra (1969) supplemented with 3% sucrose, 0.1 mg/L nephtalene acetic acid, Gamborg's vitamins and 0.8% agar, each available from Sigma, St. Louis, MO as Murashige and Skoog Based Media formula M0404, Gamborg's Based Media G5893, and Nitsch and Nitsch Media N5639, pages 127, 131, and 137 of Sigma Catalog 90, incorporated by reference herein.

Growing Conditions

Standard growing conditions were used. These were 16 hours cool white light and 25°C.

Embryo Culture

On the same media as ovules under same growing conditions as employed for the ovule culture.

Plantlet Culture

In baby food jars, on same media as embryo and ovule cultures.

Plants Produced

First Series: 1710 ovules were plated - 317 embryos were recovered - 44 developed into viable plantlets - 35 blooming plants were recovered including 18 controls and 17 putatively transformed plants.

Second Series: 1857 ovules were plated - 891 embryos were recovered - 68 developed into viable plantlets - 29 flowering plants were obtained including 11 control and 18 putatively transformed plants.

In both series, about 19% of plated ovules produced viable embryos - 10-12% of the embryos developed into plantlets, with 50% to 80% of these plantlets reaching the blooming stage and producing seeds.

Evidence of Transformation

Transformation was confirmed by histological; molecular and immunological technique.

Immunological

Extraction of β-glucuronidase was performed according to the protocol provided by CloneTech in the GUS Gene Fusion Systems User's Manual, pp. 1-5, incorporated by reference herein. This procedure employed a lysis buffer and grinding with a polytron. Identification employed a goat anti-rabbit alkaline phosphatase conjugate as secondary antibody of the rabbit anti-glucuronidase serum. Specifically, leaf extracts from putative transgenic and control plants were blotted on a nitrocellulose membrane and washed with 40 ml TSW buffer for one hour. TSW buffer comprised 10 mM Tris-Cl pH 7.4, 0.9% NaCl, 0.25% carnation instant non-fat milk, 0.1% Triton X-100, and 0.02% SDS. To the 40 ml buffer, 50 µl of rabbit antiglucuronidase serum was added. The nitrocellulose membrane was then exposed to rabbit anti-GUS serum (incubated and gentle shaking for 1 hr), then washed with 20 ml of TSW buffer for 30 min. To 50 ml TSW buffer, 5 µl goat anti-rabbit alkaline phosphatase conjugate (secondary anti-body) concentration 1 mg/ml was added and incubated with gentle shaking for 30 min. The solution was decanted and washed with 20 ml TSW buffer for 30 min. The TSW buffer was then replaced with AP development buffer [33 µl NBT (nitro blue tetrazolium) 50 mg/ml in 90% dimethyl formamide and 16.5 µl BCIP 5-bromo-4-chloro-3-indolyl-phosphate 50 mg/ml in dimethylformamide was added] and allowed to develop for

20-30 min. Purple coloration indicates presence of β-glucuronidase. Results of the test performed on plants derived from series II ovules are given in Figure 1.

Molecular

Leaf-DNA from putatively transformed and control plants was extracted according to Doyle and Doyle's technique (Focus 12(1) p. 13-15) modified as follows: an extra grinding was added after grinding in liquid nitrogen using a polytron. RNAs were eliminated using LiCl precipitation rather than RNase.

Extracted DNA was digested by Hind III and EcoRI according to standard procedure (Current Protocols in Molecular Biology, Ausubel et al. (1987), incorporated by reference herein).

Agarose gel electrophoresis of restricted DNA extracts was performed according to standard procedure also described in Current Protocols in Molecular Biology, Ausubel et al., 1987). Restricted DNA extracts were separated by electrophoresis on 0.8% agarose gel at 25 V overnight.

Southern blotting on GeneScreen Plus membrane was performed according to NEN®'s Laboratories capillary blot procedures, incorporated by reference herein. Specifically, Lambda HindIII markers (purchased from BRL, Gaithersburg, MD) served as size standards. Plain or restricted plasmid pBI221 was also included as check.

Gels were blotted onto GeneScreen Plus membrane according to the following procedure:

Incubate gel in 0.4 N NaOH - 0.6 M NaCl for 30 min. at room temperature with gentle agitation to denature DNA.

Incubate gel in 1.5 M NaCl - 0.5 M Tris-HCl, pH 7.5, for 30 min. at room temperature with gentle agitation to neutralize gel.

Cut the synthetic transfer membrane to exact size of gel.

Carefully place the synthetic transfer membrane onto the surface of a tray of deionized water. Allow the membrane to wet by capillary action.

Lay the synthetic membrane onto a 10 x SSC (1.5 M sodium chloride - 0.15 M sodium citrate) solution. Let the membrane soak for approximately 15 min.

Place a sponge into a tray or glass dish filled with 10 x SSC. The surface of the sponge, when wet, should be larger than the gel. The level of 10 x SSC should be below the top surface of the sponge.

Cut two pieces of filter paper (3 MM) to the size of the sponge.

Wet filter paper in 10 x SSC.

Place the filter paper on the sponge.

Place gel on filter paper and place gel spacers along each side of the gel.

Carefully place the synthetic membrane on the gel. Make sure that no air bubbles are trapped between the gel and the membrane.

Place 5-6 pieces of dry filter paper (cut to the same size as the gel) on top of the membrane.

Place 2-3 inch stack of absorbent paper towels (cut to the same size as the gel) on top of the filter papers.

Place a small weight on top of the paper towels.

Allow the transfer to continue for 16-24 hours.

Change paper towels frequently. Add more 10 x SSC as needed.

Carefully remove towels and filter paper without disturbing membrane.

Carefully life membrane away from gel.

Immerse membrane in an excess of 0.4 N NaOH for 30-60 sec. to ensure complete denaturation of immobilized DNA.

Remove membrane from the NaOH solution and immerse in an excess of 0.2 M Tris-HCl, pH 7.5-2 x SSC.

Place membrane with transferred DNA face up on a piece of filter paper. Allow membrane to dry at room temperature.

Upon drying, the membrane will assume its characteristic curl. To place membrane into the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the

bag, gently slide the rest of the membrane into the bag.

Membranes were hybridized for 24 hours with ^{32}p-labelled pBI221 plasmid.

The probe was prepared using Pharmacia Oligolabelling Kit and the Klenow fragment according to manufacturer instructions provided with the product. The procedure involves:

Labeling 25-50 ng of DNA to 1-2 x 10^9 dpm/μg

Dissolve the DNA in TE buffer at a concentration such that 25-50 ng can be added to the reaction in a volume of no more than 34 μl

Denature the chosen amount of DNA by heating for 2-3 min. in a water bath at 95-100°C

Immediately place the DNA on ice for 2 min., then centrifuge the tube briefly

Add the following to a clean microcentrifuge tube:

Denatured DNA (25-50 ng)	≤ 34 μl
Reagent Mix (provided)	10 μl
[α-^{32}P]dCTP (3000 Ci/mmol)	5 μl (50 μCi)
Distilled water	to total of 49 μl

Add 1 μl of Klenow Fragment. Mix gently, then centrifuge briefly

Incubate at 37°C for 30-60 min.

Specific activity averaged 1 x10^6 cpm per μg of probe.

Controls were the doubled digested plasmid (by HindIII and EcoRI), the λ ladder and the control plants (injected with TE).

Film was exposed for 48 hours at -80°C. Two obtained autoradiograms are shown in Figure 2. Plasmid was detected in a few of the putatively transformed plants: (5 in series 1, picture 2); (1 in series 2, picture 3). The latter corresponded to the plant that showed GUS expression in the previous procedure (T_{16}).

Histological

Bracts, leaf-halves or yellow petals were collected from putatively transformed and control plants, washed briefly in ethanol (100%) and rinsed in water.

They were then soaked for 48 hours in aqueous X-glucuroside solution, 50 μg/ml in 50 mM Na_3PO_4 pH 7.0 + 50 μl dimethyl formamide as described by Jefferson, Plant Molec. Biol. Rep., 5(4), 387-434 (1987), incorporated by reference herein.

Seven (T16-T19-T20-T21-T24-T25-T26) of the 18 putatively transformed plants (second series) showed the blue coloration that characterize the GUS gene expression while none of the control plants expressed that trait (Figure 3).

ANALYSIS AND OBSERVATIONS

Plantlets from each series that tested positive in one or more tests are listed in Table I. Differences in responses among tests may be due to the chimeric character of the transformed plants. Although plasmid PBI221 was injected as early as possible following fertilization inside the ovules, the developing embryos may have already included several cells and only one cell may have integrated the foreign DNA, thus producing a chimeric transgenic plant. However, these tests established that a total of 11 plants out of 34 for the two series were at least partially transgenic (tested positive in two or three tests). This corresponds to a 30% averaged success rate across both series.

Table I: Plants from series I and II that tested positive in one or more of the GUS assays.

Series	Plant No.	Fluoresc.	Immunol.	Histochem.	Molecular
I	D	-	-	-	+
	G	+	+	n.t.	+
	H	+	+	n.t.	-
	I	+	+	n.t.	+
	J	+	+	n.t.	-
	M	+	+	n.t.	-
	T1	n.t.*	-	n.t.	+
	T4	n.t.	+	n.t.	-
	T7	n.t.	+	n.t.	+
II	T12	n.t.	-	+	-
	T13	n.t.	-	+	-
	T14	n.t.	+	+	-
	T16	n.t.	-	+	+
	T18	n.t.	+	-	-
	T19	n.t.	+	-	-
	T20	n.t.	-	+	-
	T21	n.t.	+	+	-
	T23	n.t.	+	-	-
	T24	n.t.	-	+	-
	T25	n.t.	+	+	-
	T26	n.t.	+	-	-
	T27	n.t.	+	+	-

* n.t.: not tested

Effects due to medium composition were not detected since transgenic plants were obtained in roughly equal numbers on all three media tested. Although all four genotypes tested produced transgenic plants, there was some variation in the success rate depending on the genotype. Higher percentages of embryo and plant recovery from the cultured ovules were observed for cmsHA300 and cmsSDB861206, while cmsHA99 produced only a few embryos or plants. The age of the ovule also had an effect; older ovules produced more embryos and eventually more transgenic plants. Transgenic plants were obtained with all concentrations of plasmid pBI221 tested. None appeared more favorable as the most frequently used was also the most frequently successful. When averaged across genotypes, media,

ovule ages and plasmid concentrations, success rates were quite similar in both series: in series I, 166 ovules out of 821 injected with plasmid pBI221 (20%) produced an embryo, 17 of these embryos (10%) developed into mature plants of which 6 tested positive for the GUS gene in at least two assays; in series II, 878 pBI221-treated ovules produced 173 embryos (19.7%) of which 17 (9.8%) grew into mature plants, and 5 of these plants tested positive for GUS in at least two assays.

For both series, plant recovery averaged 2% of treated ovules and 30% of the derived plants were found to be transgenic. These success rates are as high as those of other transformation techniques, in particular the technique using Agrobacterium tumefaciens developed by Everett et al., Biotechnology, 5, 1201-1204 (1987) for sunflower.

Inheritance of GUS Gene in Progenies of Confirmed Transformed Plants

Progenies derived through open pollination from the transgenic plants were tested for the GUS gene using the procedure of PCR amplification and electrophoretic analysis described by McGarvey and Kaper, BioTechniques, 11(4), 428-432 (1991). The two primers, purchased from Oligos Etc. Inc., Wilsonville, OR were each a 24-nucleotide-long sequence of the GUS coding region and were separated by a 250 bp fragment of the GUS gene (Gould et al., supra., (1991), incorporated by reference herein). Presence of this fragment was revealed through electrophoresis of amplified mixtures on 1.5% gel agarose using 1 Kb DNA markers (BRL) (Figure 4). Southern Blotting hybridization on GeneScreen plus membrane according to manufacturer instructions against the p^{32}-labelled BamHI-SstI fragment of plasmid pBI221 was used to confirm the fragment identity. Inheritance of the GUS-gene in the progenies of the transgenic plants varied from 0-100% (Table II). This complete range is partly due to the highly variable number of

progenies (1 to 20 plants) recovered from each transgenic plant. Averaged across transgenic plants, inheritance frequency of the GUS-gene in the progenies was 25% (12/48). This frequency corresponds to the typical inheritance pattern of one recessive allele. According to the Southern Blots, most transgenic plants of the first generation had only introgressed a single copy of the GUS-gene, or were heterozygote recessive for that gene.

Table II. Number of progenies derived through open pollination from confirmed transgenic plants testing positive for the GUS-gene using the PCR technique.

Transgenic Plant	Number of Progenies		Inheritance Frequency(%)
	Total	Positive	
D	4	1	25
H	16	4	25
I	6	4	60
M	1	1	100
T7	1	0	0
T14	4	0	0
T16	3	0	0
T20	4	1	25
T21	4	0	0
T22	2	0	0
T27	3	2	60
T28	2	0	0

In conclusion, because of the observed Mendelian inheritance of the alien introgressed gene, transgenic lines of sunflower were derived from the application of the technique of transformation according to the invention and described herein.

WHAT IS CLAIMED IS:

1. A method for plant transformation comprising:
   (a) injecting a segment of exogenous DNA directly into the endosperm of a fertilized plant ovule at the onset of embryogenesis to introgress said DNA into the genome of said fertilized plant ovule so as to produce a transformed plant embryo.

2. The method of claim 1 wherein said DNA is injected into said ovule from about 12 hours to about 96 hours after fertilization.

3. The method of claim 1 wherein said DNA is injected into a preglobular stage plant embryo.

4. The method of claim 1 wherein said DNA is injected into a globular stage plant embryo.

5. The method of claim 1 wherein said DNA is injected into a heart-shaped stage plant embryo.

6. The method of claim 1 wherein said plant is a sunflower.

7. The method of claim 1 wherein an exogenous DNA solution of one microliter or less is injected through a tapered three microliter needle into said fertilized ovule.

8. The method of claim 1 wherein said exogenous DNA is injected into the endosperm through the butt end of said ovule about two-thirds of the way through said ovule toward said embryo.

9. The method of claim 1 wherein said exogenous DNA is a plasmid.

10. The method of claim 1 wherein said exogenous DNA is injected into said ovule as a plasmid solution.

11. The method of claim 10 wherein a 1 μl solution having about 1 μg/μl to 1 ng/μl of plasmid is injected into said ovule endosperm.

12. The method of claim 1 further comprising the step of plating injected ovules in a horizontal orientation on top of growth medium.

13. A method for plant transformation comprising:
    (a) injecting a segment of exogenous DNA encoding a desired trait directly into the endosperm of a fertilized plant ovule at the onset of embryogenesis to introgress said DNA into the genome of said fertilized plant ovule so as to produce a transformed plant embryo; and

    (b) culturing said embryo on a growth medium to develop a transformed plantlet capable of growth to a transformed plant.

14. The method of claim 13 wherein said plant is a sunflower.

15. The method of claim 13 wherein said embryo is positioned on said growth medium in a horizontal orientation.

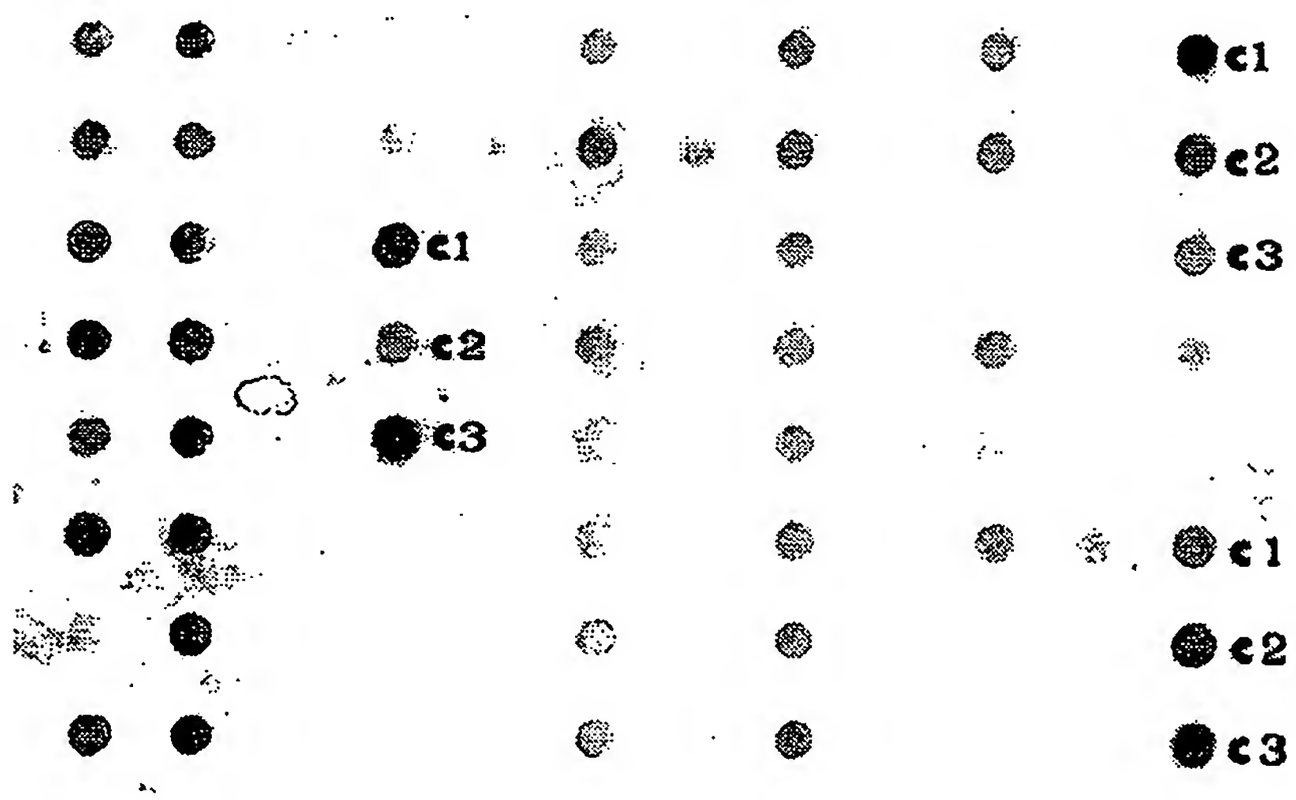
16. A method for transforming sunflowers comprising:
    (a) injecting plasmid DNA encoding a desired trait into the endosperm of a fertilized plant ovule at about 12 to 96 hours after fertilization to introgress said DNA into the genome of said fertilized plant ovule so as to produce a transformed plant embryo; and

(b) culturing said embryo on a growth medium to develop a transformed plantlet capable of growth to a transformed plant.

17. The method of claim 16 wherein said embryo is positioned in said growth medium in a horizontal orientation.

18. A transformed sunflower produced by the process of claim 16.

FIG. 1



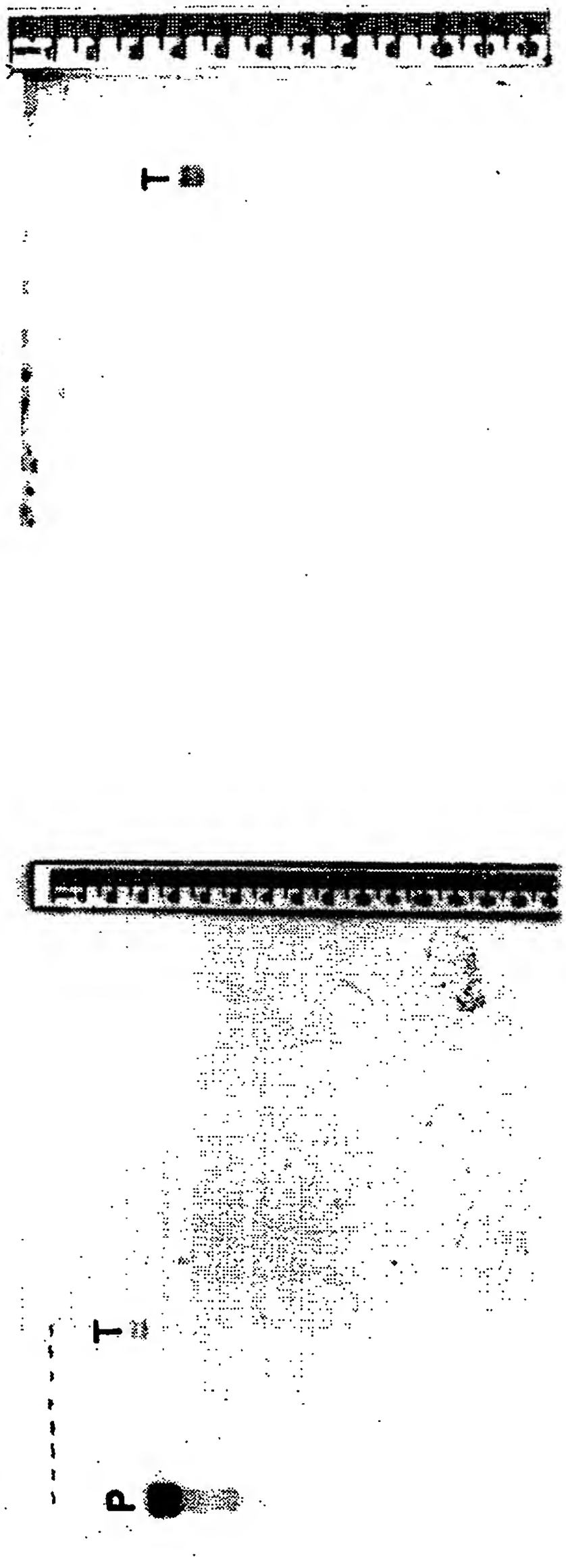


FIG. 2A

FIG. 2B

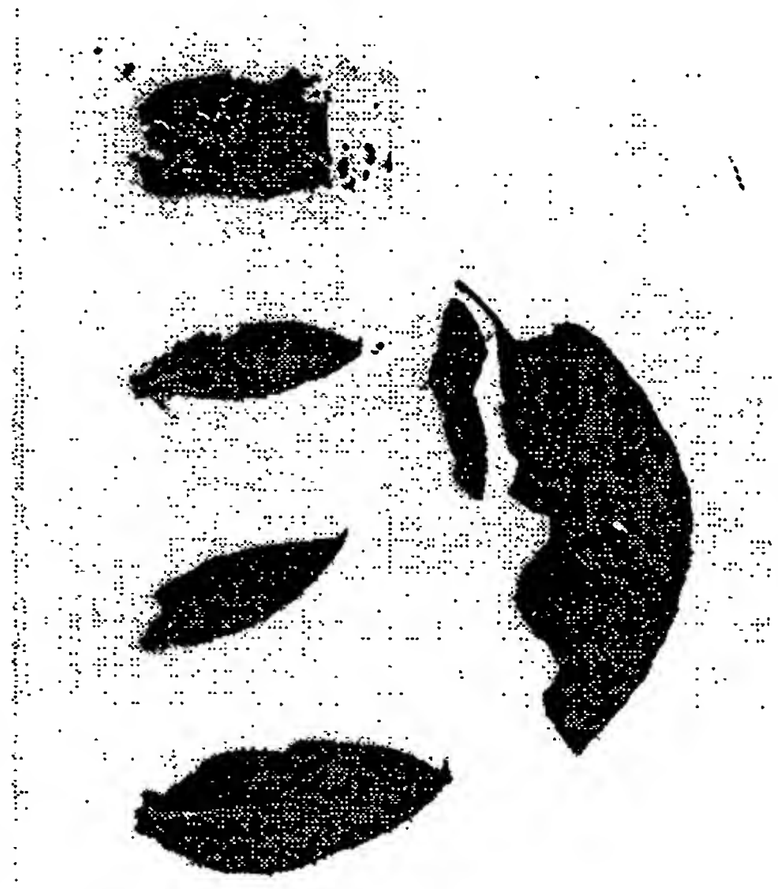


FIG. 3

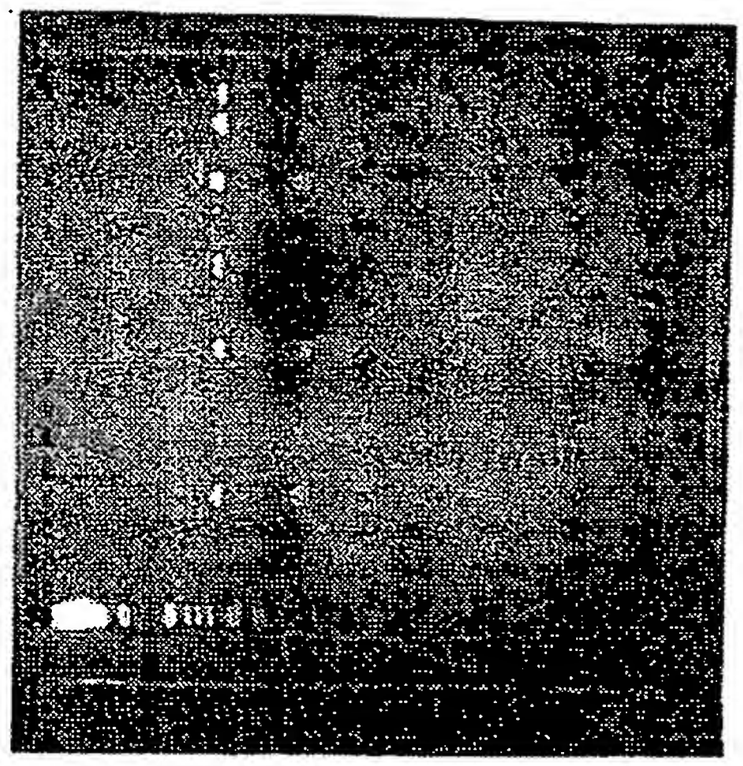


FIG. 4B

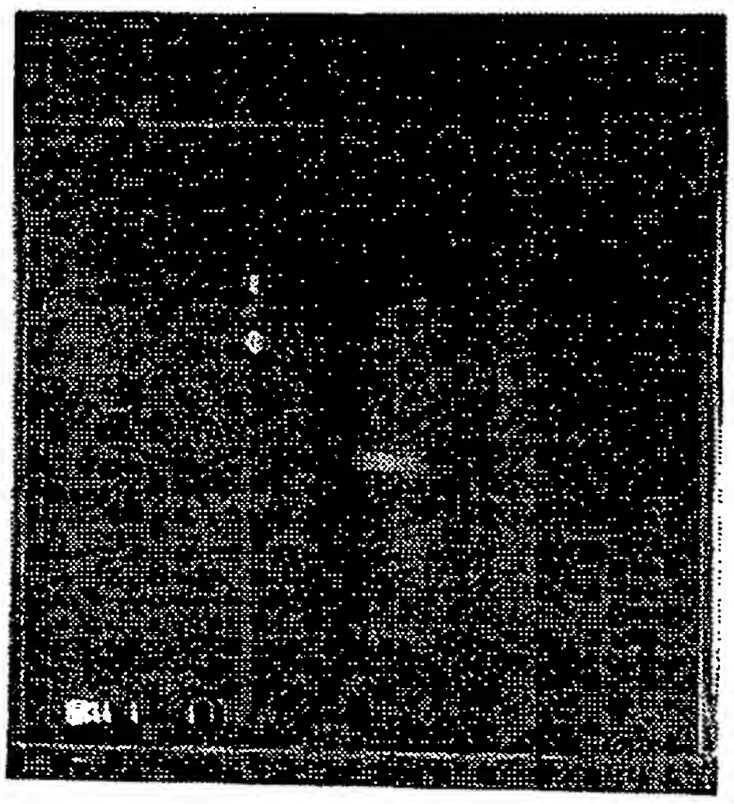


FIG. 4A

FIG. 4C

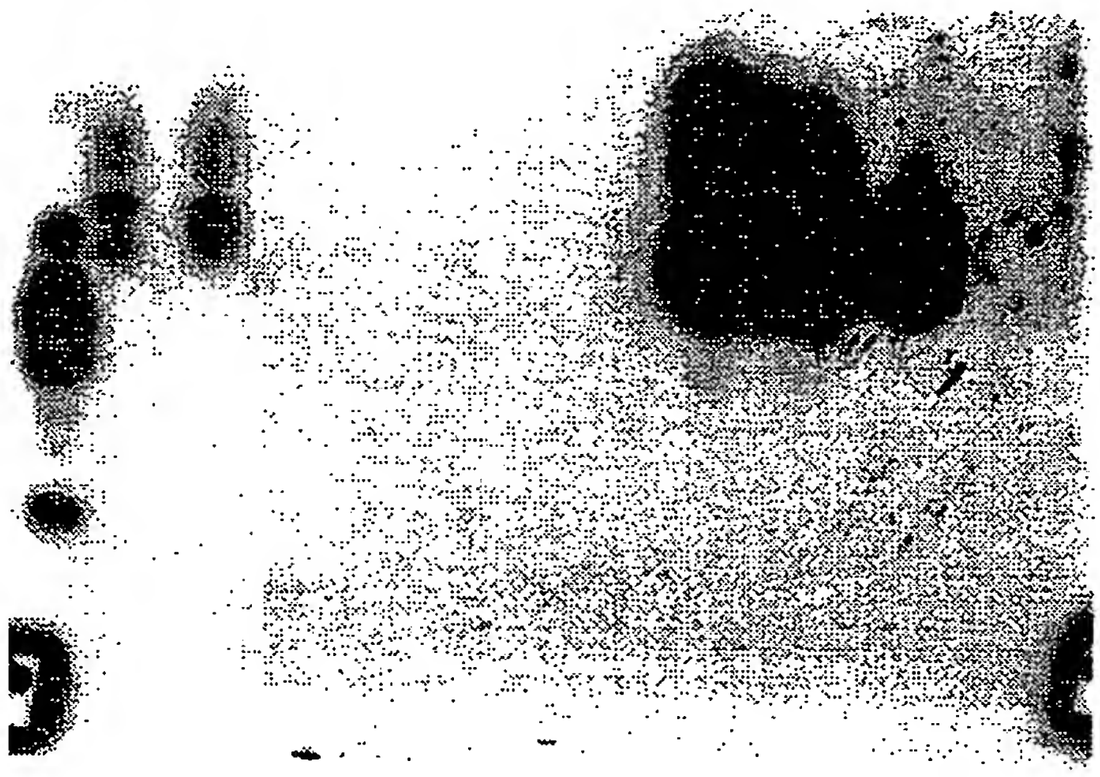
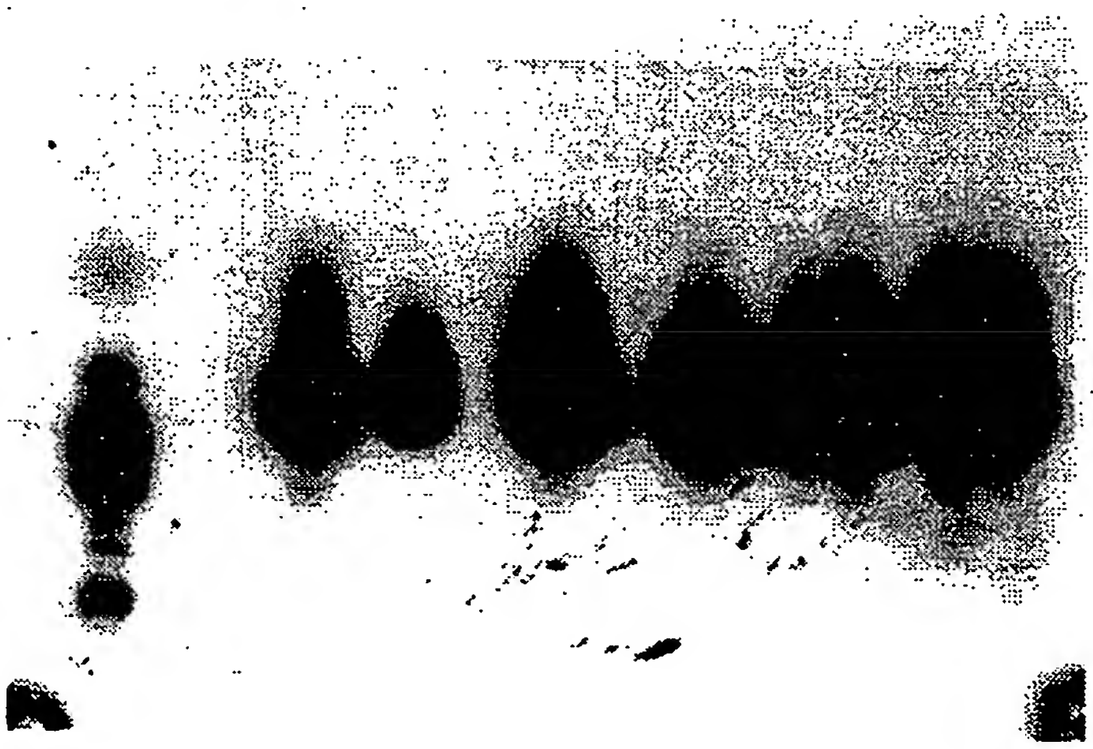


FIG. 4D



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/05825

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/89; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOTECHNOLOGY vol. 5, no. 11, November 1987, NEW YORK US pages 1201 - 1204 EVERETT, N.P., ET AL. 'Genetic engineering of sunflower (Helianthus annus L.)' see the whole document ---	18
P,X	PLANT PHYSIOLOGY. vol. 102, no. 1, May 1993, ROCKVILLE, MD, USA. page 31 REMANDE, V., ET AL. 'Transformation and regeneration systems for sunflower and soybean' see abstract 153 ---	1-18
X	EP,A,0 299 552 (SOLVAY) 18 January 1989 see page 8, line 30 - line 35 --- -/--	1,9,10, 13

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 OCTOBER 1993	14. 10. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	METHODS IN ENZYMOLOGY vol. 101, 1983, pages 433 - 481 ZHOU, G-Y, ET AL. 'Introduction of exogenous DNA into cotton embryos' see page 437 - page 438 ---	1-18
A	EP,A,0 331 083 (SCHWEIZERISCHE EIDGENOSSENSCHAFT ETH) 6 September 1989 see example 11 -----	1-18

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9305825
SA 76170

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0299552	18-01-89	NL-A- 8701450	16-01-89
		JP-A- 2000461	05-01-90
EP-A-0331083	06-09-89	AU-A- 3090489	07-09-89
		JP-A- 2016986	19-01-90